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Research Article

IN VITRO EVALUATION OF A CHIMERIC PCV1-2 CONSTRUCT IN PORCINE KIDNEY CELLS

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ABSTRACT: Porcine circovirus type 2 (PCV2) is a major causative agent of porcine circovirus-associated disease (PCVAD), responsible for significant financial losses in the swine sector. Despite the availability of commercial vaccines globally, India lacks an affordable indigenous PCV2 vaccine, with most preventive immunization dependent on imported formulations. In this study, we developed and characterized a novel chimeric PCV1-2 construct by fusing the replication-associated ORF1 of non-pathogenic PCV1 with the capsid-encoding ORF2 of PCV2. The rescued recombinant virus was successfully propagated in a subclone of porcine kidney cells, demonstrated productive replication across passages, as evidenced by gene-specific PCR, increasing capsid antigen detection by indirect ELISA (one-way ANOVA, $p<0.05$), immunofluorescence, and partial sequencing of ORF1/ORF2 confirmed the expected chimeric identity. These in vitro data indicate that the PCV1–2 construct is functional in porcine cells and suitable for further evaluation as a basis for developing an indigenous, low-cost approach to PCV2 control.

Keywords: Porcine Circovirus Type 2 (PCV2), Chimeric PCV1-2 construct, Porcine kidney cells, Capsid protein expression.

INTRODUCTION

Porcine Circoviruses (PCVs) are small, non-enveloped viruses belonging to the genus Circovirus within the family Circoviridae. These viruses are characterized by a circular, single-stranded DNA genome of approximately 1,766 to 1,768 nucleotides [1], comprising two major open reading frames (ORFs). ORF1 encodes replication-associated proteins essential for viral replication, while ORF2 encodes the immunogenic capsid (Cap) protein, which plays a critical role in capsid assembly, host cell attachment, and eliciting the host immune response [2].

Among the known PCV types, Porcine Circovirus Type 2 (PCV2) is recognized as a major pathogen in global swine production, associated with a spectrum of syndromes collectively referred to as Porcine Circovirus Diseases (PCVD) or Porcine Circovirus-Associated Diseases (PCVAD) [3]. These include respiratory illness complexes, reproductive failures, pig dermatitis and nephropathy syndrome (PDNS), and post-weaning multisystemic wasting syndrome (PMWS), and enteric disorders [4]. PCV2 is now considered globally endemic, with widespread detection across Europe, Asia, and Australia. In India, PCV2 was first reported in 2006, with subsequent outbreaks of PMWS and other PCVAD manifestations documented in various states, including Tamil Nadu, Uttar Pradesh, and Uttarakhand [5,6]. Molecular studies in India show co-circulation of multiple PCV2 genotypes principally PCV2a, PCV2b, and PCV2d, with recent datasets indicating PCV2d predominance in several regions [7,8]. Prevalence studies indicate the highest burden in the North-Eastern states, where pig farming is most intensive [9,10].

Accurate diagnosis of PCV2 infection and timely preventive measures are essential for effective disease control. A wide array of diagnostic tools, including virus

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isolation, ELISA, PCR, and real-time PCR, are employed for the detection of viral antigens and nucleic acids [11]. Serological assays such as ELISA, immunoperoxidase monolayer assay (IPMA), and virus neutralization tests are used to assess humoral responses [12].

Despite the availability of effective commercial vaccines, including inactivated, subunit, and chimeric formulations, the majority are based on the PCV2a genotype. However, increasing reports based on global data of PCV2b and PCV2d predominance raise concerns about the efficacy of PCV2a-based vaccines, which may not confer complete protection against heterologous strains, although some level of cross-protection is observed [13].

In India, the use of imported vaccines such as Ingelvac CircoFLEX is limited due to high costs, resulting in suboptimal vaccine coverage. Additionally, the development of indigenous vaccines has lagged, with most research confined to epidemiological surveillance and molecular characterization. A key barrier to developing live attenuated or inactivated PCV2 vaccines lies in the virus's poor replication in porcine cell lines and its non-cytopathogenic nature [14].

Porcine Circovirus Type 1 (PCV1), although non-pathogenic, demonstrates efficient replication in pigs and porcine cell cultures [15] and shares high genetic similarity with PCV2 [16]. Importantly, despite nucleotide and amino acid homology (approximately 80–83% and 65–67%, respectively), PCV1 does not elicit neutralizing antibodies against PCV2 [17]. These attributes render PCV1 a suitable backbone for constructing chimeric PCV1-2 viruses that integrate the immunogenic ORF2 from PCV2. Such chimeric constructs combine the safety and replicative efficiency of PCV1 with the antigenicity of PCV2, may offer a platform for affordable vaccine development [18].

Given the growing prevalence of PCV2b and PCV2d genotypes in India and the urgent need for an affordable and effective indigenous vaccine, chimeric PCV1-2 constructs present a compelling alternative. By leveraging the non-pathogenic nature and high replication efficiency of PCV1 along with the immunogenicity of PCV2 Cap protein [19], this approach may enhance protective immunity, reduce production costs, and address current gaps in vaccine availability. The present study aims to develop a chimeric PCV1-2 construct to promote self-reliance in PCV2 disease control within India. Specifically, the objective is to develop and characterize a chimeric PCV1-2 construct in porcine kidney cells.

MATERIALS AND METHODS

A PCV1-free subclone of a porcine kidney cell line, obtained from the National Centre for Cell Science (NCCS, Pune), was used in this study. Absence of PCV1 was confirmed prior to experimentation by in-house PCR using PCV1-specific primers with appropriate positive and negative controls, which yielded no amplification; the PCV1-free status of this subclone has also been reported previously [20]. Cells were propagated in Eagle's Minimum Essential Medium (EMEM; HiMedia, India) supplemented with 10% fetal bovine serum (Invitrogen, USA) and maintained under 5% CO_2.

Construction and confirmation of chimeric PCV1-2

Bulk production of recombinant pUC-PCV1-2

The synthetic PCV1-2 genome cloned in the pUC vector was transformed into chemically competent *E. coli* DH5α (Thermoscientific, USA) using the calcium chloride method. Transformants were selected on Luria Bertani (LB) agar-ampicillin (100 µg/mL) plates. Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit and verified by EcoRI digestion, followed by 1% agarose gel electrophoresis (AGE).

Cloning of PCV1-2 into pBR322

EcoRI-digested PCV1-2 genome and pBR322 vector were gel-purified and ligated using T4 DNA ligase. Ligation products were transformed into DH5α and plated on LB agar with ampicillin (100 µg/mL) and tetracycline (10 µg/mL). Colonies were screened via colony PCR using PCV1-2 specific primers, and positive clones were miniprepped and confirmed by 1% AGE.

Transfection and propagation in PK-15 Cells

Transfection of recombinant pBR322-PCV1-2 was performed in a subclone of PK-15 cells using Lipofectamine® 3000. After 12 hours post-transfection, the media was replaced with MEM + 5% FBS. Cells were harvested after 72 hours post-infection (P1 stock). Four blind passages were conducted; the recombinant pBR322-PCV1-2 was propagated further up to the 8th passage.

Virus confirmation

PCR and indirect ELISA

DNA was extracted from passages 4, 6, and 8 using the HiPurA DNA Kit. PCR was performed using PCV1-2 F forward and PCV1-2R reverse primers to confirm the chimeric virus genome with amplification conditions (35 cycles of 94 °C for 30 s, 55 °C for 60 s,

72 °C for 60 s, followed by final extension at 72 °C for 10 min. Chimeric PCV1-2 genome expression was also evaluated via indirect ELISA using PCV2 monoclonal Antibody (Invitrogen) and HRPO-conjugated secondary antibody. OD was measured at 450 nm.

Immunofluorescence and confocal microscopy

Subclones of PK-15 cells were infected with rescued virus and were fixed and stained using fluorescein isothiocyanate (FITC)-tagged anti-PCV2 polyclonal serum (VMRD). Nuclei and cytoskeleton were counterstained using DAPI and Actin Red, respectively. Images were captured using FlouView 1000 Confocal laser scanning biological microscope (Olympus, Japan) at 60X magnification.

Sequencing

Partial ORF1 and ORF2 regions were sequenced (Eurofins, Bengaluru). Sequences were aligned and analyzed using MEGA 11 software.

Statistics

OD_{450} values across passages were compared by one-way ANOVA with Tukey's post-hoc test (GraphPad Prism v9). Normality/homoscedasticity were verified; when assumptions were not met, Kruskal–Wallis with Dunn's correction was applied. $\alpha = 0.05$

RESULTS AND DISCUSSION

Construction and confirmation of recombinant pUC vector containing PCV1-2 genome

EcoRI digestion of the recombinant constructs released the expected ~1.76 kb insert, as visualized by electrophoresis on a 1% agarose gel (Fig. 1). The released insert was subsequently purified and quantified.

Construction and confirmation of recombinant pBR-322 vector

The pBR-322 vector was linearized with EcoRI and ligated with the EcoRI-digested PCV1-2 genome. The recombinant pBR322/PCV1–2 was recovered from DH5α cells. EcoRI digestion released the expected 1,762 bp insert, confirming correct cloning (Fig. 2). Purified plasmid preparations yielded 1.5 µg/µL DNA.

Table 1. Oligonucleotide primer used for the confirmation of the chimeric PCV1-2 genome.

Primer	Sequence (5' → 3')	Amplicon size (bp)
A (PCV1-2F)	GAGCAGTTCCCTGTAACGTATG	819
B (PCV1-2R	TCCTACCACTCCCGCTATTT	

Transfection and passaging of chimeric PCV1-2 in porcine kidney cells

The recombinant pBR-322/PCV1-2 plasmid was transfected into porcine kidney cells. Successful transfection and viral replication were confirmed by PCR using gene-specific primers (Fig. 3), direct fluorescent antibody assay (DFA), indirect ELISA with PCV2 capsid-specific monoclonal antibody, and partial sequencing.

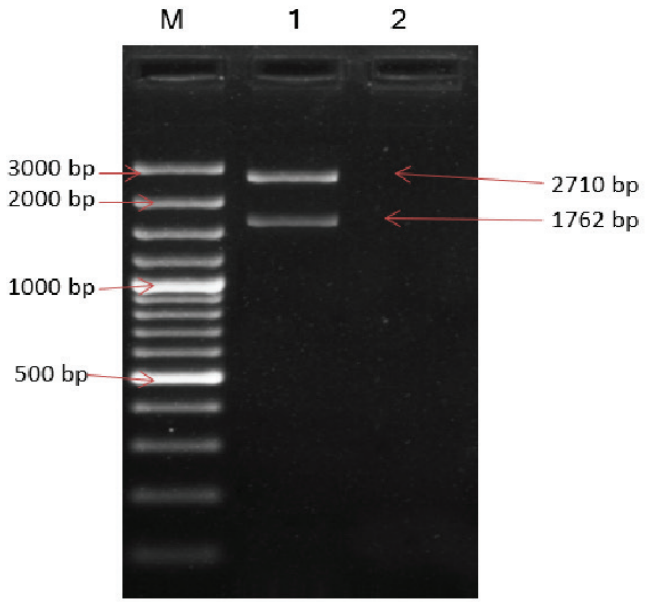


Fig. 1. R E digestion of PUC/PCV1-2. [Lane M- Next gen 3kbp ladder; Lane 1-Released product; Lane2- No template control].

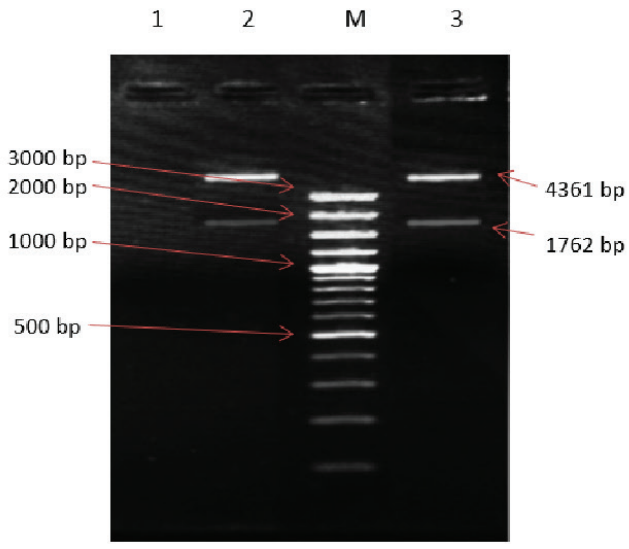


Fig. 2. R E digestion of PBR322/PCV1-2. [Lane1- No template control; Lane 2- Released product from colony 9; Lane M - Next gen 3kbp ladder; Lane 3- Released product from colony 13].

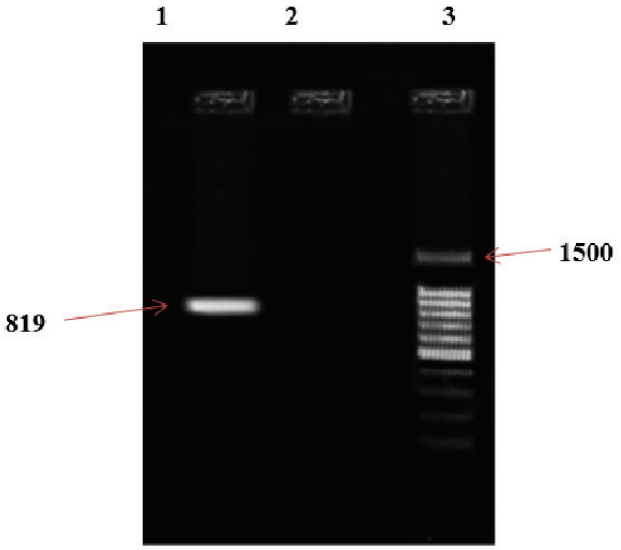


Fig. 3. R E digestion of PBR322/PCV1-2. [Lane1- No template control; Lane2- Released product from colony 9; Lane M- Next gen 3kbp ladder; Lane3- Released product from colony 13].

Expression assessment by indirect cell ELISA

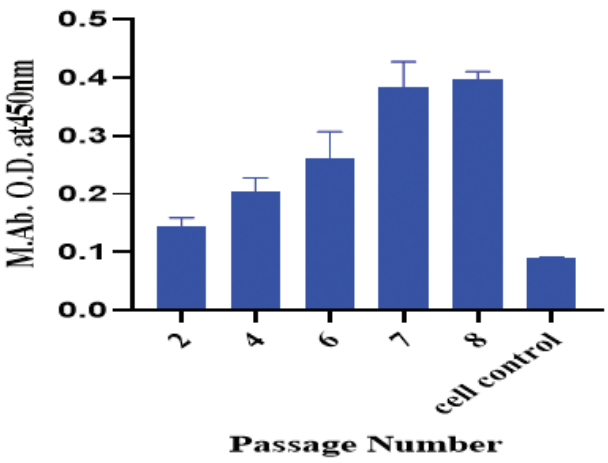
Indirect ELISA showed a progressive increase in OD at 450 nm (OD_{450}) from passages 4 to 8, indicating rising viral antigen production. OD_{450} values for all passages were significantly higher than the cell control. One-way ANOVA detected overall group differences ($p < 0.05$), and post-hoc pairwise comparisons confirmed significant increases between successive passages (Fig. 4a, b).

Confocal microscopy for viral capsid protein

Green fluorescence (capsid protein) was observed with DAPI (blue, nucleus) and actin red staining (cytoskeleton). In infected cells, green fluorescence was observed along with a blue nuclear stain and red coloured cytoskeleton, indicating expression of the capsid protein of the rescued recombinant PCV1-2 virus. While in uninfected control cells, only a blue nuclear stain and red colored cytoskeleton were observed**.** (Fig. 5).

Different passage number of chimeric virus	Mean O D
2	0.143
4	0.205
6	0.262
7	0.382
8	0.396
Cell control	0.095

(A)



(B)

Fig. 4. Indirect ELISA for chimeric virus cell lysate at different passage number. (A) Mean OD values of chimeric virus at various passages. (B) Bar graph showing increased antigen expression with higher passage numbers; cell control used as negative control.

Cytopathic effect

No overt cytopathic effect was observed during serial passaging, consistent with the generally non-cytopathic nature of PCV infections in PK-15 cells [21]

Sequencing of partial regions of rescued virus at passage 6

Partial sequencing analysis at passage level 6 confirmed alignment with the reference genome through BLAST and chromatogram validation (Fig. 6A and B). The presence of partial ORF1 (PCV1) and partial ORF2 (PCV2) regions was verified. As only partial ORF1/ORF2 segments were analyzed, we do not infer whole-genome stability from these data.

Porcine circovirus–associated disease (PCVAD) due to PCV2 continues to impose measurable productivity losses. In England (pre-vaccine baseline, 2008), the national cost was estimated at £52.6 million/year rising to ~£88 million/year during epidemic years (2002-2005); per-pig losses ranged from £8.1 (PCV2-subclinical survivors) to £84.1 (PMWS deaths), and at the farm level the largest share of impact was attributed to

subclinical PCV2 infection [22, 23]. Subclinical PCV2 infection depresses average daily gain in proportion to viral load, compounding hidden productivity losses [24]. In India, robust nationwide monetized estimates are not yet available; however, with a national pig population of ~9.06 million (2019 Livestock Census) and widespread PCV2 circulation, the economic burden is likely substantial, underscoring the need for sustained vaccination and monitoring [25-26].

Currently available commercial vaccines, primarily based on the PCV2a genotype, have demonstrated efficacy in reducing viremia, mortality, and improving production parameters under both experimental and field conditions [27, 28, 29]. However, recent epidemiological data reveal a genotypic shift, with PCV2b and PCV2d now predominating in various regions, including India. Although PCV2a-based vaccines offer partial cross-protection, their reduced

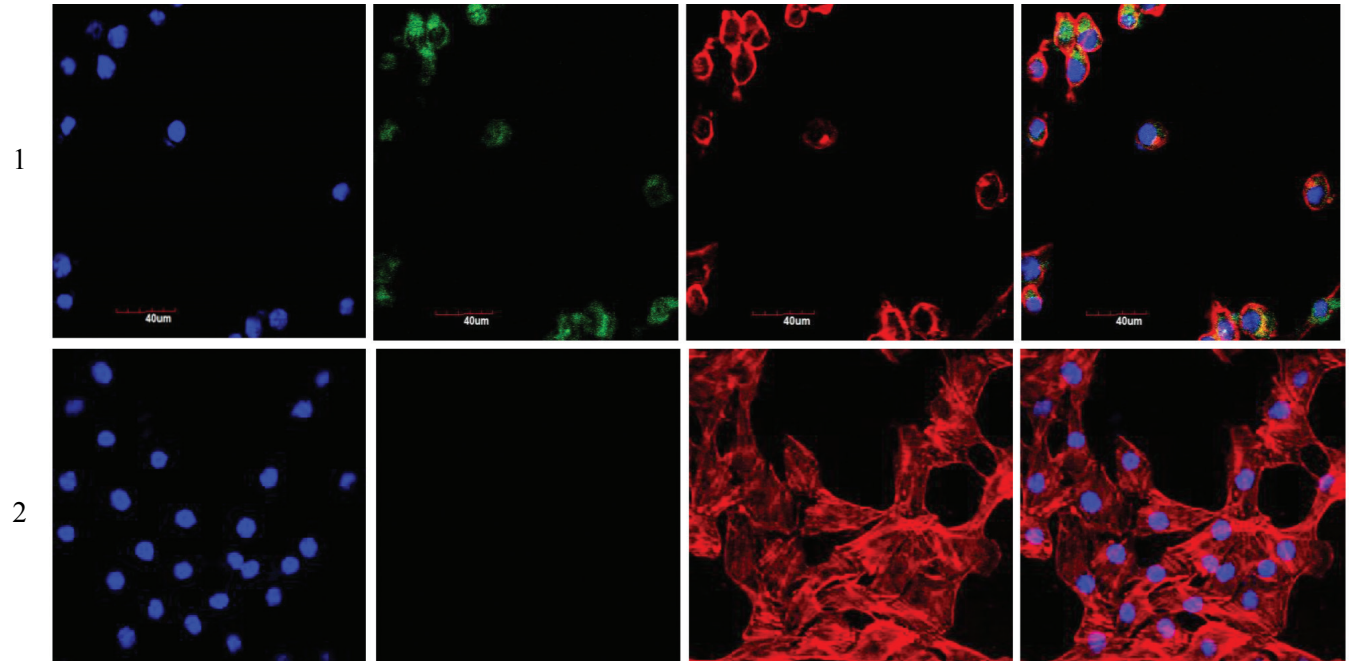
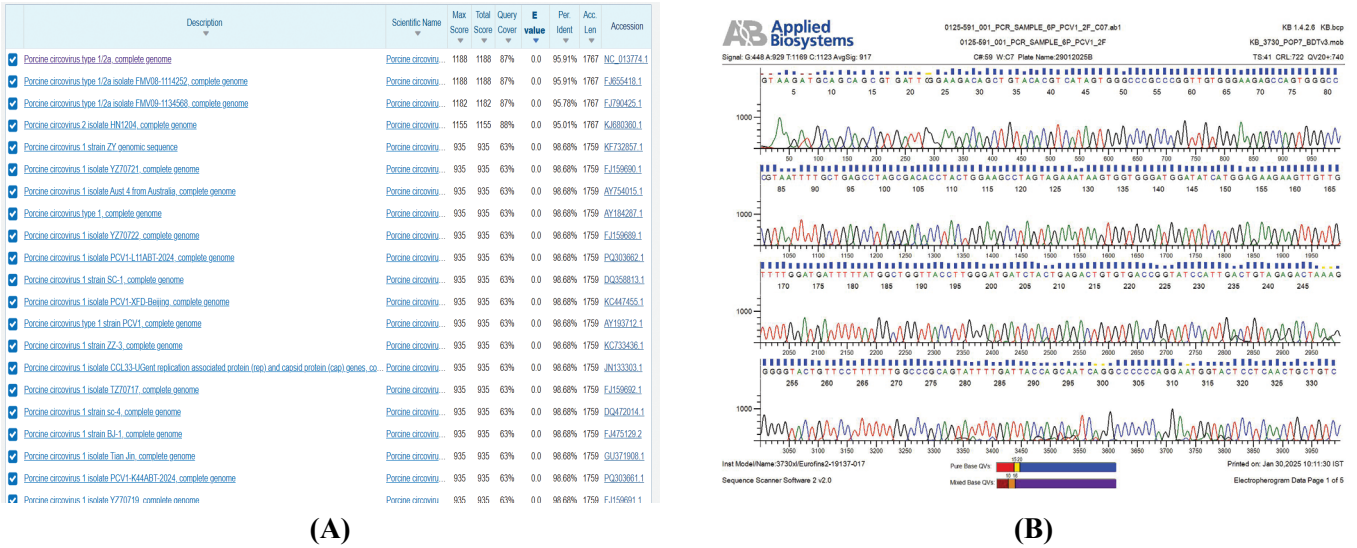


Fig. 5. Confocal microscopy at 60x magnification using FITC tagged Commercial antibodies, DAPI for nuclear stain and actin red stain for cytoskeleton. [1. Chimeric virus infected sub clone of porcine kidney cells, 2. Uninfected control sub clone of porcine kidney cells, a.DAPI stained nucleus after excitation, b. FITC monitored for capsid protein after excitation, c.Actin red stained cytoskeleton after excitation.


Description
Scientific Name
Max Score
Total Score
Query Cover
E value
Per. Ident
Acc. Len
Accession
Porcine circovirus type 1/2a, complete genome
Porcine circovirus type 1/2a isolate FMV08-1114252, complete genome
Porcine circovirus type 1/2a isolate FMV09-1134568, complete genome
Porcine circovirus 2 isolate HN1204, complete genome
Porcine circovirus 1 strain ZY genomic sequence
Porcine circovirus 1 isolate YZ70721, complete genome
Porcine circovirus 1 isolate Aust 4 from Australia, complete genome
Porcine circovirus type 1, complete genome
Porcine circovirus 1 isolate YZ70722, complete genome
Porcine circovirus 1 isolate PCV1-I-11ABT-2024, complete genome
Porcine circovirus 1 strain SC-1, complete genome
Porcine circovirus 1 isolate PCV1-XFD-Beijing, complete genome
Porcine circovirus type 1 strain PCV1, complete genome
Porcine circovirus 1 strain ZZ-3, complete genome
Porcine circovirus 1 isolate CCL33-UGent replication associated protein (rep) and capsid protein (cap) genes, co...
Porcine circovirus 1 isolate TZ70717, complete genome
Porcine circovirus 1 strain sc-4, complete genome
Porcine circovirus 1 strain BJ-1, complete genome
Porcine circovirus 1 isolate Tian Jin, complete genome
Porcine circovirus 1 isolate PCV1-K44ABT-2024, complete genome
Porcine circovirus 1 isolate YZ70719, complete genome
Applied Biosystems
0125-591_001_PCR_SAMPLE_6P_PCV1_2F_C07.ab1
0125-591_001_PCR_SAMPLE_6P_PCV1_2F
KB_3730_POP7_BDTv3.mob
Sequence Scanner Software 2 v2.0
Printed on: Jan 30,2025 10:11:30 IST
Electropherogram Data Page 1 of 5

(A) **(B)**

Fig. 6. Sequence confirmation of rescued PCV1-2 virus at passage level 6 in porcine kidney cell subclone. [A. NCBI BLAST alignment confirming identity with the reference genome; B. chromatogram for sequence validation].

efficacy against heterologous strains, especially PCV2b and PCV2d, raises concerns about long-term vaccine effectiveness and highlights the need for next-generation vaccines with broader cross-protective potential [30].

In response to this need, the present study focused on the development and characterization of a recombinant chimeric PCV1-2 construct containing the capsid gene (ORF2) from a prevalent PCV2b strain, inserted into the genomic backbone of non-pathogenic PCV1. PCV1-2 chimeric construct presents several advantages over conventional inactivated or subunit vaccines. PCV1 is non-pathogenic in swine, allowing for safe replication and antigen presentation without inducing disease [31, 32, 33]. ORF2 encodes the major capsid protein, which is the primary target of neutralizing antibodies and is critical for eliciting protective immunity [34-35]. The substitution of ORF2 from PCV2b into the PCV1 framework aims to combine the safety profile of PCV1 with the immunogenicity of field-relevant PCV2 strains, providing a rational approach to broaden the vaccine's protective efficacy [36].

The chimeric PCV1-2 genome was successfully constructed and cloned initially into pUC57, followed by cloning into the low-copy-number pBR322 vector. Restriction enzyme digestion confirmed the integrity of the insert, consistent with previously reported molecular cloning approaches for circular viral genomes [37, 38].

Transfection of a subclone of PK-15 cells with the chimeric construct using Lipofectamine 3000 facilitated the successful rescue of the recombinant virus. The presence of the rescued virus across serial passages (P4, P6, and P8) was confirmed via PCR using chimeric genome-specific primers. This was further validated by indirect ELISA using cell lysates and a monospecific anti-PCV2 capsid antibody, which showed increasing OD values indicative of active viral replication. These findings are consistent with earlier reports demonstrating the successful rescue of recombinant PCV1-2 viruses using similar methodologies [39].

Antigenic expression of the chimeric virus was assessed by direct immunofluorescence. The transfected subclone of PK-15 cells exhibited specific cell-associated fluorescence across serial passages, consistently above background in mock-transfected and secondary-only controls. Signal evaluation was qualitative; fluorescence intensity was not quantified. These findings confirm successful viral replication and expression of the PCV2b-derived capsid protein. No fluorescence was detected in uninfected control cells, supporting assay specificity [40]. At passage 6, partial regions of ORF1 and ORF2 were Sanger-sequenced. BLASTn analyses showed ≥99% nucleotide identity to the expected references (PCV1 for ORF1; PCV2b for ORF2) [41]. Chromatograms exhibited clean, single peaks without mixed bases, insertions, or deletions. These data confirm the intended genomic elements and indicate sequence stability in the regions examined; whole-genome stability was not assessed.

Despite the wide availability of commercial vaccines globally, PCV2 vaccination in India is limited to a few organized farms, primarily due to the high cost of imported vaccines such as Ingelvac CircoFLEX. Indigenous vaccine development is thus essential to ensure accessibility, affordability, and sustainability in swine health management in the country. This study represents a significant step toward that goal, offering foundational data for the future development and in vivo evaluation of a recombinant, chimeric PCV1-2-based vaccine tailored to circulating Indian PCV2 strains.

CONCLUSION

In this study, a chimeric PCV1–2 construct was rescued and characterized in vitro in porcine kidney cells. The construct replicates productively across passages with detectable capsid antigen and expected partial-region sequence identity. These findings support advancing to in vivo evaluation of safety, immunogenicity, and protective efficacy toward an indigenous approach to PCV2 control in India.

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